JOURNAL OF VIROLOGY, July 1973, p. 13-17
Vol. 12, No. 1

Printed in U.S.A.

Isolation and Properties of *Bacillus subtilis* Strains Lysogenized by a Clear Plaque Mutant of Bacteriophage ϕ105

ANTHONY J. GARRO

Department of Microbiology, Mount Sinai School of Medicine, The City University of New York, New York, New York 10029

Received for publication 5 January 1973

A clear plaque mutant of the temperate *Bacillus* phage ϕ105 lysogenized a small fraction of infected cells forming an integrated prophage at or near the normal ϕ105 insertion site. These lysogens exhibited a spontaneous induction rate approximately 1,000-fold lower than wild type and were noninducible (*ind*$^-$) by mitomycin C. Prophage was induced, however, when competent cultures were incubated with transforming DNA. The *ind*$^-$ phenotype could not be attributed solely to the clear plaque mutation and appears to involve a cell-specific factor. Lysogenization by the clear plaque mutant, in contrast to wild-type phage, did not cause a marked reduction in transformation efficiency.

The plaques formed by temperature phages are turbid due to growth of lysogenized bacterial cells within the plaque. Phage mutants which form clear plaques usually are defective in their ability to lysogenize infected cells. This phenotype is not restricted to mutants which never lysogenize but also includes mutants whose lysogenic potential is impaired but not completely eliminated (8).

The temperate *Bacillus* phage ϕ105 lysogenizes strains of *B. subtilis* 168 forming an integrated inducible prophage (3, 13). Several clear plaque mutants of ϕ105 have been described (2, 13); two of these, cc1 and cts23, are temperature-sensitive mutants, whereas the c4 mutant forms clear plaques at 30 and 40 C. The cts23 gene product is necessary for the maintenance of repression; prophage carrying this mutation are temperature inducible. The gene function(s) defined by the cc1 and c4 mutations are not known.

This report describes the isolation and properties of cells lysogenized by ϕ105c4. It is apparent from this study that the c4 gene product is not the ϕ105 repressor.

MATERIALS AND METHODS

Bacteria and phage. The bacterial strains used are listed in Table 1. Stocks of ϕ105, including the spontaneous clear plaque mutant ϕ105c4 (13), were kindly supplied by L. Rutberg.

Media and growth of phage and bacteria. Phage ϕ105 was assayed for PFU on Min-CH overlay plates (13). To enumerate free phage in the presence of ϕ105-infected or -lysogenized cells, this medium was supplemented with 5 mg of streptomycin sulfate per ml, and samples were plated with a streptomycin-resistant indicator, SR135 Str^r.

The defective phage, PBSX, was titered by spotting lysates on overlay plates consisting of 2.5 ml of the top agar described by Okubo and Romig (11), seeded with the PBSX-sensitive strain *B. subtilis* W23M8 (6), layered onto Tryptose blood agar base (TBAB). PBSX titers are expressed as the reciprocal of the highest dilution which inhibits growth of the indicator. Because ϕ105 does not produce plaques on *B. subtilis* W23 strains (3), the presence of this phage in lysates does not interfere with the PBSX assay.

Veal-yeast extract (VY) broth contained 2.5% veal infusion (Difco) and 0.5% yeast extract (Difco). Bacterial colony-forming units were determined by streaking dilutions on TBAB plates.

Isolation of ϕ105-resistant and ϕ-105c4-lysogenic

TABLE 1. *Bacterial strains*

Strain	Relevant properties[a]	Source
BR95	*phe-1, ilvA-1, try-2*	L. Rutberg
SR135	*try-7, spoA-9, su*$^+$3	L. Rutberg
MB54	*metC*	J. Marmur
W23 M8	PBSX^s	J. Marmur
SR135 Str^r	*try-7, spoA-9, su*$^+$3, *str*^{r}	Our collection

[a] Properties: *spoA-9*, asporogenous; *su*$^+$, suppresses the *try-7* mutation; PBSX^s, killed by the defective phage PBSX (14); *str*^{r}, resistant to 5 mg of streptomycin sulfate per ml.

strains. Exponentially growing cultures in Min-CH medium were infected with ϕ105c4 at a multiplicity of 3 to 5. Following lysis, an equal volume of fresh medium was added to the cultures and incubation was continued for 16 h to enrich for cells which could grow in the presence of the phage. These cells were cloned on TBAB plates and purified several times by picking and restreaking single colonies. To screen for ϕ105 resistance, or immunity, cultures of these clones in Min-CH medium were tested for their ability to grow across a streak of ϕ105c4 (6×10^9 PFU/ml) on a TBAB plate.

Bacterial transformation and mapping. Bacteria lysogenized by ϕ105c4 were grown to competence and transformed with DNA isolated from a wild-type ϕ105 lysogen, and prototrophic recombinants were selected and purified as described previously (6). Linkage between the c4 mutation and bacterial markers was determined from the plaque phenotype of phage spontaneously liberated by purified transformed clones cultured in VY broth.

RESULTS

Isolation of ϕ105c4 lysogens. The first indication that c4 mutants of ϕ105 were able to lysogenize followed an attempt to use this phage as a selective agent for the isolation of ϕ105-resistant mutants of *B. subtilis*. One isolate, thought to be a resistant strain, was observed to produce plaques when overlaid in the presence of ϕ105-sensitive cells. Subsequent examination revealed that several other clones, initially classified as ϕ105 resistant, spontaneously liberated a clear plaque phage. Two of these, SR135(ϕ105c4) and BR95(ϕ105c4), were selected for further study.

Adsorption of ϕ105. The failure of ϕ105 to produce plaques on lawns of SR135(ϕ105c4) or BR95(ϕ105c4) is not due to an inability to adsorb the phage. Within 15 min after the addition of ϕ105 to these cultures, between 80 and 90% is adsorbed (Table 2).

Chromosomal location of ϕ105c4 prophage. Since the presence of ϕ105c4 in cultures of SR135(ϕ105c4) and BR95(ϕ105c4) could be indicative of either a carrier state (16) or true lysogeny, it was necessary to determine whether the ϕ105c4 genome was integrated into the host chromosome.

Rutberg (13) has demonstrated that the insertion site for ϕ105 prophage lies between the host markers *phe* and *ilv*, and that prophage determinants are cotransformed with either *phe* or *ilv* with a frequency of approximately 10% (12). To investigate if linkage could be demonstrated between the c4 determinant and either of these markers, BR95(ϕ105c4) was transformed with DNA isolated from MB54(ϕ105), and *phe*$^+$, *ilv*$^+$, and *try*$^+$ transformants were selected. Transformants which had undergone recombination for prophage genes were detected by examining the plaque phenotype of spontaneously liberated phage. The results presented in Table 3 demonstrate that the c4 mutation cotransforms with *phe* and *ilv*, indicating that ϕ105c4 forms an integrated prophage at or near the normal ϕ105 insertion site.

Two classes of prophage recombinants were observed. One class results in the conversion from a clear to a turbid plaque type phage and accounts for 9% of the *ilv*$^+$ and 13% of the *phe*$^+$ transformants. These recombinants appear to have acquired the c4$^+$ allele from the wild-type prophage. The second class, which is observed less frequently, has lost the ability to produce infectious phage but has retained ϕ105-specific immunity. Recombinants in this class may have lost a phage gene(s) essential for lytic development and appear to be analogous to the defective lysogens described for coliphage λ (1, 9). No recombinants were observed which produced both clear and turbid plaque type phages, suggesting that double lysogens are not formed.

Contrasts between ϕ105 and ϕ105c4 lysogens. Cells lysogenized by ϕ105c4 differ from those lysogenized by wild-type ϕ105 with respect to their inducibility and efficiency of transformation. The rate of spontaneous induc-

TABLE 2. *Absorption of ϕ105*[a]

Strain	Fraction of phage unadsorbed[b]
BR95	0.14
BR95(ϕ105c4)	0.20
SR135(ϕ105c4)	0.10

[a] Cultures growing exponentially in Min-CH, 37 C, were infected at a multiplicity of 0.5 to 1.0 with ϕ105.

[b] Ratio of the number of PFU due to free phage remaining unadsorbed at 15 min to the number of PFU added.

TABLE 3. *Prophage recombinants produced by transformation*[a]

Selected marker	No. tested	Prophage phenotype		
		Clear	Turbid	Defective[b]
ilvA-1	198	174	17	7
phe-1	200	169	27	4
try-2	200	198	2	0

[a] BR95 (ϕ105c4) was transformed with nonsaturating concentrations of DNA (0.01 μg/ml) isolated from MB54 (ϕ105).

[b] Recombinants in this class did not liberate infectious phage but retained immunity to infection by ϕ105.

tion of ϕ105c4 prophage (Table 4), measured in cultures growing exponentially in VY broth, was found to be 10^3 to 10^4 lower than that reported for wild-type ϕ105 (3). Furthermore, formation of infectious ϕ105c4 is not induced when the lysogenic strains are exposed to mitomycin C (MC). An increased production of infectious phage is observed, however, when ϕ105c4 lysogenic cultures are grown to competence and exposed to transforming DNA (Table 5). In agreement with the results reported by Peterson and Rutberg (12), this effect was not observed with cells lysogenized by wild-type phage. The phenomenon of DNA-mediated prophage induction will be discussed in a subsequent paper.

Bacteria lysogenic for ϕ105 transform at greatly reduced levels relative to nonlysogenic strains (12, 17). This effect was not observed with the ϕ105c4 lysogenic derivative of BR95 (Table 6). The frequency of transformation of BR95(ϕ105c4), though somewhat reduced compared to the nonlysogenic strain, is several hundred times greater than that of the wild-type lysogen. The transformation efficiency of SR135(ϕ105c4) was not examined since these cells carry the *su*$^+$3 suppressor and are phenotypically *try*$^+$.

Characteristics of ϕ105c4$^+$ recombinants. As a consequence of the mapping study described above, it was possible to isolate recombinants of BR95(ϕ105c4) which liberate turbid plaquing phage (Table 3). All of these recombinants, which are designated BR95T(ϕ105c4$^+$), retain the low level of spontaneous phage production characteristic of ϕ105c4 lysogens. Furthermore, when tested for transformation to *try*$^+$ each of 20 randomly picked BR95T(ϕ105c4$^+$) clones, generated by selecting either *phe*$^+$ or *ilv*$^+$ recombinants of BR95 (ϕ105c4), exhibited the high transformation efficiency of the parental strain, BR95(ϕ105c4). There are two possible explanations for this. (i) The phage ϕ105c4 carries a mutation in addition to the one responsible for its clear plaque phenotype, which contributes to the induction and transformation characteristics of the ϕ105c4 lysogens. (ii) These characteristics are due to some cell-specific factor.

It was possible to differentiate between these possibilities by lysogenizing bacteria with the phage liberated by a BR95T(ϕ105c4$^+$) recombinant and examining their phenotype. To isolate the desired lysogens, BR95T(ϕ105c4$^+$) was cultured for 16 h in VY broth, and the medium, which contained free phage, was sterile-filtered and plated in overlays seeded with BR95. The centers of the turbid plaques produced were picked, and the lysogens, designated BR95(ϕ105c4$^+$), were cloned on TBAB plates. As seen in Table 7 these lysogens exhibit the induction and transformation characteristics of wild-type ϕ105 lysogenized bacteria, indicating that the phenotype of the original ϕ105c4 lysogens is cell specific.

TABLE 4. *Induction of prophage*

Strain	Spontaneous (frequency/ bacterial generation)	MC[a]	
		Yielders[b]	Burst
SR135(ϕ105)	1.3×10^{-5}	1.4	100
BR95(ϕ105)	1.7×10^{-4}	2	78
SR135(ϕ105c4)	$<1 \times 10^{-8}$	1×10^{-4}	3×10^{-6}
BR95(ϕ105c4)	$<1 \times 10^{-8}$	4×10^{-4}	$<1 \times 10^{-6}$

[a] Mitomycin C (MC) induction: cultures growing exponentially in VY broth (2×10^7 cells/ml) were treated with 0.4 μg of MC per ml. Lysis occurred within 120 min.

[b] Yielders: ratio of infectious centers, after MC addition but before burst, to viable cells present before MC.

TABLE 5. *Effect of transforming DNA[a] on phage production*

Strain	Induction ratio (DNA/buffer)[b]
SR135(ϕ105c4)	47
BR95(ϕ105c4)	35
SR135(ϕ105)	1
BR95(ϕ105)	1

[a] MB54 DNA, 4 μg/ml.

[b] These values represent the ratio of PFU due to free phage measured at 120 min after the addition of either DNA or buffer (0.015 M NaCl, 0.0015 M trisodium citrate, pH 7) to competent cultures.

TABLE 6. *Relative frequencies of transformation[a]*

Strain	Marker selected		
	ilvA-1	*phe-1*	*try-2*
BR95	1.0	1.0	1.0
BR95(ϕ105c4)	0.4	0.4	0.5
BR95(ϕ105)	1×10^{-3}	1×10^{-3}	1×10^{-3}

[a] Competent cultures were transformed with saturating concentrations of DNA (4 μg/ml) isolated from MB54. The frequency of transformation (transformants/total viable cells) for each culture was calculated, and the values obtained were divided by the frequency observed for the nonlysogenic strain.

Induction of PBSX from ϕ105c4 lysogens. All strains of *B. subtilis* 168 carry the inducible defective phage PBSX (14, 15). It was possible, by examining the inducibility of PBSX, to determine if the factor which causes the induc-

TABLE 7. *Characteristics of ϕ105c4$^+$ lysogens*

Strain	Induction[a]			Transformation	
	Spontaneous (frequency/ bacterial generation)	MC		*try-2* transformants[b] per 10^8 cells	R[c]
		Yielders	Burst		
BR95T(ϕ105c4$^+$)	$<1 \times 10^{-8}$	0.6×10^{-4}	$<1 \times 10^{-6}$	2.5×10^5	2.9×10^2
BR95(ϕ105c4$^+$)	9×10^{-5}	2.4	26	8.5×10^2	

[a] Induction measured in VY broth.
[b] MB54 DNA (4 μg/ml) was added to competent cultures.
[c] Ratio of transformation efficiencies BR95T(ϕ105c4$^+$):BR95(ϕ105c4$^+$).

tion-minus (*ind*$^-$) phenotype of ϕ105c4 lysogens also affects this phage. Exponentially growing cultures of SR135(ϕ105c4) and BR95(ϕ105c4) in VY broth were treated with MC (0.4 μg/ml), and the time course of lysis and PBSX production was followed. As seen in Fig. 1, PBSX is inducible from the ϕ105c4 lysogens, indicating that the *ind*$^-$ phenotype is specific for the c4 prophage. Further proof of this specificity was provided by the finding that the temperate *Bacillus* phage SPO2 lysogenizes and is inducible from BR95(ϕ105c4) and the BR95T(ϕ105c4$^+$) recombinant (data not shown).

DISCUSSION

The principal conclusion which can be drawn from the experiments reported here is that the gene function inactivated by the c4 mutation is not required for prophage maintenance or immunity. Although it is apparent that the c4 gene product is not the ϕ105 repressor, the role of c4 function in the establishment of lysogeny remains to be resolved.

The *ind*$^-$ phenotype of ϕ105c4 lysogens cannot be attributed solely to the c4 mutation since transformants which have acquired the c4$^+$ determinent retain this characteristic. Interestingly, all λ*ind*$^-$ mutants which have been described are associated with the cI gene which codes for the λ repressor (4, 7). However, no evidence was found for an additional ϕ105 mutation to explain the *ind*$^-$ phenotype, i.e., the phage released from *ind*$^-$ c4$^+$ recombinants such as BR95T(ϕ105c4$^+$) behave like wild-type ϕ105 when transferred to a new host (Table 7).

The cell-specific nature of the *ind*$^-$ phenotype may be explained by either (i) a cellular mutation which may have been selected for by the procedures used to isolate the c4 lysogens, or (ii) the formation of an aberrant prophage which is not efficiently induced to form infectious phage. If ϕ105c4 forms a prophage which is aberrant in either its exact integration site or gene order, then the resulting inhomologies which would occur during recombination with wild-type ϕ105 lysogen DNA could account for the generation of defective prophage seen in the mapping experiment (5).

Both explanations are compatible with the finding that PBSX and SPO2 are inducible from ϕ105c4 lysogens. A cellular mutation which is specific for ϕ105 may reflect the need for a host function during some step unique to ϕ105 induction. In this regard Armentrout and Rutberg (2) have recently demonstrated that, in contrast to phages such as λ and P22, several steps are involved in separating ϕ105 prophage from bacterial genes during induction. PBSX induction, on the other hand, does not appear to require prophage excision (10), whereas SPO2

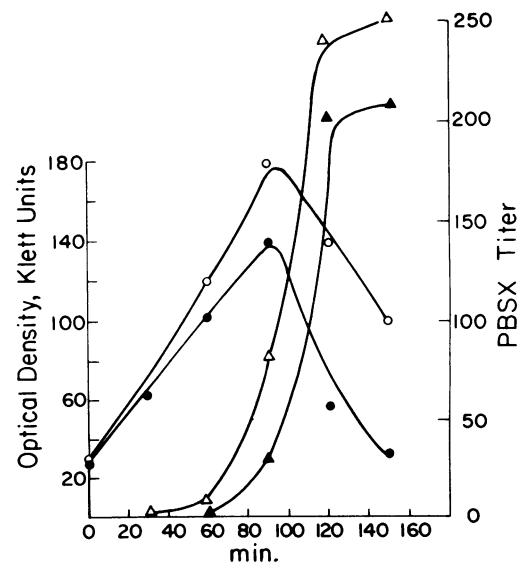

FIG. 1. *Kinetics of PBSX induction. MC (0.4 μg/ml) was added at 0 min to cultures growing exponentially in VY broth at 37 C. Optical density (OD) was followed in a Klett colorimeter at 670 nm. PBSX titers were determined as described in Materials and Methods. SR135(ϕ105c4): OD(●), PBSX(▲); BR95(ϕ105c4): OD(○), PBSX(△).*

induction has not been studied in sufficient detail to comment upon.

The last relationship to be considered is the effect of lysogenization on transformation and transfection. The fact that ϕ105c4 lysogens are transformed with an efficiency that is several hundredfold greater than that of wild-type lysogens (Tables 6 and 7) may be related to their low level of spontaneous induction. Preliminary experiments have indicated that competent cells of wild-type lysogens are exceptionally prone to spontaneous induction (Garro, unpublished observation). Since transformation requires continued cell viability and induction results in cell death, a high level of spontaneous induction would result in fewer potential transformants in competent cultures. Transfection, on the other hand, does not require continued cell viability, and, though Peterson and Rutberg (12) reported lysogenization to decrease transfection by ϕ1 DNA, Yasbin and Young (17) have reported transfection by ϕ29 and SPO1 to be unaffected.

ACKNOWLEDGMENTS

I thank Viera Honzova for her expert technical assistance.

This work was supported by grant no. VC-38 from the American Cancer Society.

LITERATURE CITED

1. Appleyard, R. K. 1954. Segregation of new lysogenic types during growth of a doubly lysogenic strain derived from *Escherichia coli* K12. Genetics **39**:440-452.
2. Armentrout, R. W., and L. Rutberg. 1971. Heat induction of prophage ϕ105 in *Bacillus subtilis*: replication of bacterial and bacteriophage genomes. J. Virol. **8**:455-468.
3. Birdsell, D. C., G. M. Hathaway, and L. Rutberg. 1969. Characterization of temperate *Bacillus* bacteriophage ϕ105. J. Virol. **4**:264-270.
4. Eshima, M., S. Fujii, and T. Horiuchi. 1972. Isolation of λind^- mutants. Jap. J. Gen. **47**:125-128.
5. Franklin, N. C. 1971. Illegitimate recombination, p. 175-194. *In* A. D. Hershey (ed.), The bacteriophage λ. Cold Spring Harbor, New York.
6. Garro, A. J., H. Leffert, and J. Marmur. 1970. Genetic mapping of a defective bacteriophage on the chromosome of *Bacillus subtilis* 168. J. Virol. **6**:340-343.
7. Jacob, F., and A. Campbell. 1959. Sur le systèm de repression assurant l'immunité chez la bacteriés lysogenes. C. R. Acad. Sci. Paris **248**:3219-3221.
8. Kaiser, A. D. 1957. Mutations in a temperate bacteriophage affecting its ability to lysogenize *Escherichia coli*. Virology **3**:42-61.
9. Lederberg, E. M., and J. Lederberg. 1953. Genetic studies of lysogenicity in *Escherichia coli*. Genetics **38**:51-64.
10. Okamoto, K., J. A. Mudd, J. Morgan, W. M. Huang, T. V. Subbaiah, and J. Marmur. 1968. Properties of the defective phage of *Bacillus subtilis*. J. Mol. Biol. **34**:413-428.
11. Okubo, S., and W. R. Romig. 1965. Comparison of ultraviolet sensitivity of *Bacillus subtilis* bacteriophage SPO2 and its infectious DNA. J. Mol. Biol. **14**:130-142.
12. Peterson, A. M., and L. Rutberg. 1969. Linked transformation of bacterial and prophage markers in *Bacillus subtilis* 168 lysogenic for bacteriophage ϕ105. J. Bacteriol. **98**:874-877.
13. Rutberg, L. 1969. Mapping of a temperate bacteriophage active on *Bacillus subtilis*. J. Virol. **3**:38-44.
14. Seaman, E., E. Tarmy, and J. Marmur. 1964. Inducible phages of *Bacillus subtilis*. Biochemistry **3**:607-613.
15. Subbaiah, T. V., C. D. Goldthwaite, and J. Marmur. 1965. Nature of bacteriophages induced in *Bacillus subtilis*, p. 435-446. *In* V. Bryson and H. J. Vogel (ed.), Evolving genes and proteins. Academic Press Inc., New York.
16. Takahashi, I. 1964. Incorporation of bacteriophage genome by spores of *Bacillus subtilis*. J. Bacteriol. **87**:1499-1502.
17. Yasbin, R. E., and F. E. Young. 1972. The influence of temperate bacteriophage ϕ105 on transformation and transfection in *Bacillus subtilis*. Biochem. Biophys. Res. Commun. **47**:365-371.